From the Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

MOLECULAR ADAPTATIONS OF SKELETAL MUSCLE IN HEALTH AND DISEASE

Maja Schlittler

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MOLECULAR ADAPTATIONS OF SKELETAL MUSCLE IN HEALTH AND DISEASE

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Maja Schlittler

Principal Supervisor:
Docent Daniel C. Andersson
Karolinska Institutet
Department of Physiology and Pharmacology
Translational cardiac and skeletal muscle physiology

Co-supervisor:
Professor Håkan Westerblad
Karolinska Institutet
Department of Physiology and Pharmacology
Cellular muscle physiology

Opponent:
Associate Professor Nikolai B. Nordsborg
University of Copenhagen
Department of Nutrition, Exercise and Sports
Integrative Physiology

Examination Board:
Professor Eva Blomstrand
Swedish School of Sport and Health Sciences
Åstrand Laboratory

Professor Fawzi Kadi
Örebro University
School of Health Sciences

Professor Harriet Wallberg
Karolinska Institutet
Department of Physiology and Pharmacology
Integrative Physiology

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ABSTRACT

Appropriate function of skeletal muscle is essential for locomotion, everyday activities and athletic performance. In addition to its mechanic tasks, skeletal muscle communicates with other organs via metabolic pathways and regulatory processes. Skeletal muscle is a plastic tissue that adapts to external stimuli, including hormonal signalling, exercise, physical inactivity and prolonged disuse. Molecular adaptations at the muscle fibre level have local effects on force-production and fatigue-resistance. In addition, they can alter metabolic pathways and regulatory processes with effects on whole-body physiology. The aim of this thesis was to study local and systemic effects of molecular adaptations of skeletal muscle in physiologic and pathologic conditions.

In paper I, we investigated the consequences of muscular adaptations to sprint interval training (SIT) on contractile force. We demonstrate that a single session of SIT induces modifications of the ryanodine receptor (RyR1) in untrained humans and that repeated exposure to SIT provides some protection from SIT-induced RyR1 modifications. We moreover show that a three-week SIT program improves exercise performance but does not accelerate recovery of neuromuscular function after SIT.

In the second paper, we studied molecular adaptations of skeletal muscle in a mouse model of amyotrophic lateral sclerosis (ALS), a neuromuscular disease that causes denervation and muscle weakness. The aim of this study was to determine whether muscle weakness in ALS is caused by the degeneration of motor neurons and subsequent atrophy or whether muscle fibre intrinsic defects (ie, altered Ca^{2+} handling or altered contractile properties) contribute to the loss of contractile force. Muscles of symptomatic ALS mice exhibited motor neuron loss, atrophy and reduced absolute force. However, at the single fibre level, Ca^{2+} handling was preserved, force-generating capacity intact and fibres displayed endurance training-like adaptations with increased fatigue-resistance and signs of mitochondrial biogenesis. Hence, surviving muscle fibres of ALS mice were strong and adaptable and muscle weakness was caused by muscle atrophy and not by muscle fibre intrinsic defects.

Papers III and IV looked at molecular adaptations of skeletal muscle that have systemic effects on regulatory and metabolic pathways. In paper III, we studied skeletal muscles of humans lacking the structural protein α-actinin-3 (ACTN3) due to a common null polymorphism in the *ACTN3* gene. The lack of ACTN3 has undergone positive selection in recent evolution and seems to provide a survival advantage in cold areas potentially linked to increased skeletal muscle Ca^{2+} cycling. In our study, humans with ACTN3 deficiency showed improved cold-resilience when exposed to an acute cold-challenge in conjunction with a shift in the expression of the Ca^{2+} handling proteins SERCA and calsequestrin. In addition, we observed altered muscle fibre distribution in ACTN3 deficient subjects with an increased proportion of type I and a decreased proportion of type IIx fibres. In summary, ACTN3 deficient subjects are more efficient at maintaining their body temperature during acute cold exposure potentially linked to an increased proportion of type I muscle fibres.

In paper IV, we looked at molecular adaptations of skeletal muscle in response to endurance exercise that affect peripheral kynurenine (KYN) metabolism and cross-talk between muscle and brain. The degradation of KYN to NAD^+ produces neurotoxic metabolites, which have been associated with depression. In an alternative pathway, KYN is converted to the neuroprotective kynurenic acid (KYNA) and this process is catalysed by kynurenine aminotransferases (KATs). Recent animal studies have shown that endurance exercise increases the expression of KATs in skeletal muscle resulting in a shift of the peripheral KYN metabolism towards the neuroprotective branch, thereby protecting from stress-induced depression. In our study, we show that healthy humans who participate in regular endurance training have increased expression of skeletal muscle KATs. We moreover demonstrate that acute endurance exercise increases the flux through the neuroprotective branch of the KYN pathway resulting in a transient increase of circulating KYNA. In contrast, high-intensity eccentric exercise did not affect circulating KYN metabolites. In summary, our study shows that prolonged, metabolically demanding exercise alters peripheral KYN metabolism which may have bearing on training recommendations for patients with depression.

Taken together, the four studies presented in this thesis underline the multifacetedness of skeletal muscle as a tissue and the implications of molecular adaptations for athletic performance and for chronic disease.

LIST OF SCIENTIFIC PAPERS

I. **Maja Schlittler***, Daria Neyroud*, Christian Tanga, Nadège Zanou, Sigitas Kamandulis, Albertas Skurvydas, Bengt Kayser, Håkan Westerblad, Nicolas Place[#], Daniel C. Andersson[#]. *Three weeks of sprint interval training improved high-intensity cycling performance and limited ryanodine receptor modifications in recreationally active human subjects.* Eur J Appl Physiol. 2019 Jun 27.
[*, #] *equal contribution*

II. Cheng AJ, Allodi I, Chaillou T, **Schlittler M**, Ivarsson N, Lanner JT, Thams S, Hedlund E, Andersson DC. *Intact single muscle fibres from SOD1^{G93A} amyotrophic lateral sclerosis mice display preserved specific force, fatigue resistance and training-like adaptations.* J Physiol. 2019 Jun;597(12):3133-3146.

III. Wyckelsma VL*, **Schlittler M***, Venckunas T, Brazaitis M, Ivarsson N, Paulauskas H, Eimantas N, Andersson DC, Westerblad H. *Superior cold-resilience in α-actinin-3 deficient individuals is linked to a higher proportion of type I muscle fibres.* Manuscript
[*] *equal contribution*

IV. **Schlittler M**, Goiny M, Agudelo LZ, Venckunas T, Brazaitis M, Skurvydas A, Kamandulis S, Ruas JL, Erhardt S, Westerblad H, Andersson DC. *Endurance exercise increases skeletal muscle kynurenine aminotransferases and plasma kynurenic acid in humans*. Am J Physiol Cell Physiol. 2016 May 15;310(10):C836-40.

CONTENTS

LIST OF ABBREVIATIONS

ACTN2	α-actinin-2
ACTN3	α-actinin-3
$ACTN3^{-/-}$	α-actinin-3 knockout mouse strain
ALS	Amyotrophic lateral sclerosis
$[Ca^{2+}]_i$	Cytosolic free $[Ca^{2+}]$
CaM	Calmodulin
CTRL	Group of recreationally active control subjects
DHPR	Dihydropyridine receptors
EC coupling	Excitation-contraction coupling
END	Group of endurance trained subjects
EMG	Electromyography
FDB	Flexor digitorum brevis
FKBP12	FK506-binding protein
3HK	3-hydroxykynurenine
IDO	Indoleamine 2,3-dioxygenase
KATs	Kynurenine amino transferases
KYN	Kynureine
KYNA	Kynurenic acid
MVC	Maximal voluntary contraction
NAD^+	Nicotinamide adenine dinucleotide
NDMA	N-methyl-D-aspartate
P50 / P125 / P150	Mice at 50 / 125 / 150 days of age
PGC-1α1	Peroxisome proliferator-activated receptor γ coactivator-1α1
P_i	Inorganic phosphate
PLFFD	Prolonged low-frequency force depression
PPARα	Peroxisome proliferator-activated receptor alpha
PPARδ	Peroxisome proliferator-activated receptor delta
PS10 / PS100	Supramaximal paired electrical stimulation pulses at 10 Hz / 100 Hz
QUIN	Quinolinic acid
ROS	Reactive oxygen species
RR	Group with 577RR genotype
577RR	Homozygotes for the *ACTN3* ‘wild type’ allele

RyR1 / RyR2 / RyR3	Ryanodine receptor 1 / 2 / 3
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
SIT	Sprint interval training
SOD1	Superoxide dismutase 1
$SOD1^{G93A}$	Mouse strain overexpressing mutated human SOD1
SR	Sarcoplasmic reticulum
TDO	Tryptophan 2,3-dioxygenase
T_{mu}	Intramuscular temperature of the gastrocnemius muscle
T_{re}	Rectal temperature
T_{sk}	Skin tempeature
t-tubules	Transverse tubular system
VAL	Voluntary activation level
Wmax	Wattmax / maximal power reached during incremental test
XX	Group with 577XX genotype
577XX	Homozygotes for a null polymorphism in the *ACTN3* gene

1 INTRODUCTION

The over 600 muscles in the human body generate force for posture control, locomotion, movement and respiration. In a contracting muscle fibre, cytosolic free Ca^{2+} is the main regulator of contractile force. The first part of this introduction addresses the general function of skeletal muscle fibres with a specific focus on the movement of intracellular Ca^{2+} between the sarcoplasmic reticulum and the cytosol and its implications for force production. Further, alterations of cytosolic Ca^{2+} concentrations at rest and during contractions will be discussed in the context of muscle fatigue following intense exercise, and in muscle weakness associated with a neuromuscular disorder.

In addition to its force-generating tasks, skeletal muscle is involved in a range of metabolic and signalling pathways with both, local and systemic effects (Pedersen and Febbraio 2012). Accounting for more than one third of the human body, skeletal muscle is the main energy-consuming tissue, which has implications on metabolic pathways and thermoregulation (Janssen et al. 2000). The second part of the introduction will illustrate on two different examples how local changes in skeletal muscle can affect whole-body physiology. Specifically, it will describe *i*) how the lack of a muscle protein may contribute to thermogenesis and provide a beneficial adaptation in cold environments and *ii*) how skeletal muscle participates in metabolic pathways related to mental health.

1.1 SKELETAL MUSCLE FUNCTION

1.1.1 Excitation-contraction coupling

The activation of a skeletal muscle cell is initiated by an electrical impulse in the cortex that travels along the spinal cord and then via the axon of a lower motor neuron to the neuromuscular junction where it triggers the release of the neurotransmitter acetylcholine. Binding of acetylcholine to receptors in the postsynaptic membrane facilitates the influx of Na^{+}, depolarization of the sarcolemma, and hence the initiation of an action potential (Allen et al. 2008b). From the neuromuscular junction, the action potential spreads along the surface of the sarcolemma and is further conducted into the muscle fibre via the transverse tubular system (t-tubules), a network of narrow invaginations of the sarcolemma (Stephenson et al. 1998). Each t-tubule is surrounded by two terminal cisternae of the sarcoplasmic reticulum (SR) forming the structural basis for the transformation of the electrical signal to mechanical force (excitation-contraction coupling; EC coupling) (Franzini-Armstrong 1970). The membrane of the t-tubule contains voltage-sensitive L-type Ca^{2+} channels (dihydropyridine receptors; DHPR) that are in close contact with the ryanodine receptor 1 (RyR1), the Ca^{2+} release channel in the SR membrane (Calderon et al. 2014; Paolini et al. 2004; Zalk et al. 2015). In cardiac muscle, EC coupling is mediated by the influx of extracellular Ca^{2+} through the DHPR whereas activation of SR Ca^{2+} release in skeletal muscle occurs through physical contact between the DHPR and the RyR1, independent of extracellular Ca^{2+} (Bers 2002; Lamb 2000). Depolarization of the t-tubule induces a conformational change in the DHPR,

which opens the RyR1 via allosteric interaction (Schneider and Chandler 1973). At rest, there is a large gradient of free Ca^{2+} between the SR and the cytosol (0.3-1 mM *vs.* ~70-80 nM) and hence, opening of the RyR1 results in a fast release of Ca^{2+} from the SR and a marked increase of cytosolic free [Ca^{2+}] ($[Ca^{2+}]_i$) (Allen et al. 2008b).

The contractile machinery of a skeletal muscle fibre consists of actin and myosin filaments, which interact via cross-bridge generation. In the relaxed state, cross-bridge formation is disabled because the myosin binding sites on the actin filaments are covered by the regulatory protein tropomyosin. During EC coupling, Ca^{2+} released by the SR binds to troponin C, which displaces tropomyosin from the myosin-binding sites thereby enabling attachment of the myosin heads to the actin filament (Melzer et al. 1995; MacIntosh et al. 2012). In an ATP-dependent process, the myosin heads tilt and slide the actin filaments past the myosin filaments producing contractile force (Huxley 1969). After the so-called "power stroke", the myosin heads detach, rotate back into their original position and reattach to a new active binding site. Cross-bridge cycling continues as long as enough Ca^{2+} is available. For muscle relaxation, Ca^{2+} ions are pumped back into the SR by the ATP-consuming SR Ca^{2+}-ATPase (SERCA). As $[Ca^{2+}]_i$ decreases, troponin and tropomyosin return to their initial conformation and the muscle relaxes (Calderon et al. 2014).

1.1.2 Regulation of contractile force

The contractile force of a skeletal muscle fibre during isometric contraction depends largely on the diameter of the fibre and therefore specific force (ie, contractile force normalized to the cross-sectional area) is typically used to allow for comparisons between muscle fibres. Specific force is primarily determined by the number of simultaneously active cross-bridges, which, in turn depends on the number of Ca^{2+} ions bound to troponin C and hence on $[Ca^{2+}]_i$ (MacIntosh et al. 2012). Normally, the relationship between contractile force and $[Ca^{2+}]_i$ follows a sigmoidal curve with almost no force generation when $[Ca^{2+}]_i$ is close to resting levels, a steep linear part where small changes in $[Ca^{2+}]_i$ result in large changes of force, and a plateau phase where troponin C is saturated with Ca^{2+} and contractile force is maximal (MacIntosh et al. 2012; Westerblad and Allen 1996). Experimentally, the force-$[Ca^{2+}]_i$ relationship of a muscle fibre can be determined by electrical stimulation with varying frequencies, which provides different tetanic $[Ca^{2+}]_i$ concentrations with their corresponding force values (Fig. 1A) (Allen et al. 2008a). Conditions, such as neuromuscular diseases, pharmaceutical treatments or muscle fatigue change the force-$[Ca^{2+}]_i$ relationship by altering the sensitivity of the myofibrils for Ca^{2+} and/or the force produced by the individual cross-bridges (Allen et al. 1989; Westerblad and Allen 1996, 1993). A decreased myofibrillar Ca^{2+} sensitivity results in reduced force for a given $[Ca^{2+}]_i$, which manifests as a right-shift of the force-$[Ca^{2+}]_i$ curve, whereas changes in the cross-bridge force-generating capacity become apparent in the plateau phase of the curve (Fig. 1B) (Westerblad and Allen 1996; MacIntosh et al. 2012).

During muscle contraction, $[Ca^{2+}]_i$ increases transiently from a resting level of 50-100 nM to an average tetanic $[Ca^{2+}]_i$ of approximately 1 µM in mammalian fast-twitch muscle

fibres (Dahlstedt and Westerblad 2001; Aydin et al. 2009; Yamada et al. 2015). The magnitude and time-course of the $[Ca^{2+}]_i$ rise depend to a large extent on the movement of intracellular Ca^{2+} between the SR and the cytosol, ie, the rate of Ca^{2+} release by the RyR1, the rate of Ca^{2+} reuptake via SERCA and the amount of Ca^{2+} buffered by troponin C and the cytosolic Ca^{2+}-buffer parvalbumin (Westerblad and Allen 1996). To a minor degree, entrance of extracellular Ca^{2+} over the sarcolemma and uptake of Ca^{2+} by the mitochondria may contribute to changes in $[Ca^{2+}]_i$, however, these effects are more pronounced during long term experiments and are negligible in acute fatigue under physiological conditions (Cho et al. 2017; Launikonis et al. 2010; Bruton et al. 2003). Altered Ca^{2+} handling (ie, SR Ca^{2+} release, SR Ca^{2+} reuptake and Ca^{2+} buffering) is one important cause of decreased contractile force during muscle fatigue and in different pathological conditions and will be discussed in the following sections.

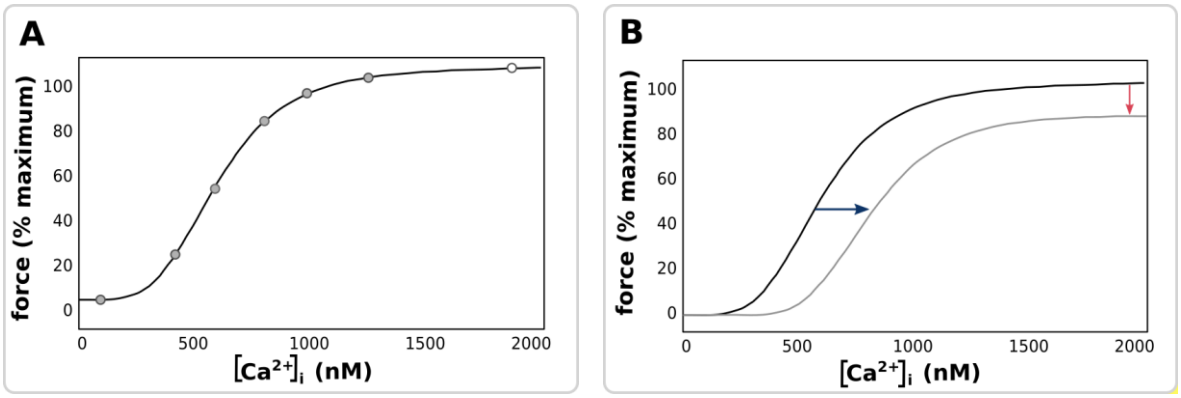

Figure 1. Force-$[Ca^{2+}]_i$ relationship of an isolated muscle fibre. (A) Force-$[Ca^{2+}]_i$ relationship of an unfatigued single muscle fibre. The grey circles indicate force and $[Ca^{2+}]_i$ values for contractions of the same fibre at different stimulation frequencies. The white circle marks the maximal $[Ca^{2+}]_i$ and corresponding force induced by the application of caffeine. **(B)** Decreased myofibrillar Ca^{2+} sensitivity results in a right-shift of the force-$[Ca^{2+}]_i$ relationship (blue arrow) whereas decreased cross-bridge force-generating capacity is visible in the plateau phase of the curve (red arrow). Figures adapted from (Allen et al. 2008a) and (Cheng et al. 2018).

1.1.3 Muscle fatigue and recovery

Fatigue is characterized by a decline in voluntary contractile force, typically induced by repeated activation of a muscle (Allen et al. 2008b). Fatigue can arise during any of the steps along the path of muscle contraction and is referred to as central fatigue if it originates from a decreased neuronal drive or as peripheral fatigue, if it is caused by changes at the level of the muscle (Gandevia 2001; Allen et al. 2008b). During natural activities, such as walking or running, muscles are activated by short, repeated contractions and fatigue is mainly determined by peripheral factors (Allen et al. 2008b). Hence, this section will focus on the contribution of molecular changes to fatigue at the single cell level.

During repeated contractions, tetanic $[Ca^{2+}]_i$ and force of a muscle fibre follow a typical pattern with three distinct phases: during the first few contractions, force declines by ~10-

20% while tetanic $[Ca^{2+}]_i$ increases. This initial phase is followed by a relatively long period where tetanic $[Ca^{2+}]_i$ and force remain almost constant while the final phase is characterized by a rapid drop of both, tetanic $[Ca^{2+}]_i$ and force (Allen et al. 2008b; Westerblad and Allen 1993; Place et al. 2009). The force decrease in the first phase of fatigue is attributed to a decrease in myofibrillar force-generating capacity whereas decreased contractile force in the final phase is the combined result of reduced tetanic $[Ca^{2+}]_i$ and decreased myofibrillar Ca^{2+}-sensitivity (Allen et al. 2008a; Place et al. 2009; Cheng et al. 2018).

The alterations in cellular Ca^{2+} handling observed during fatigue are primarily a consequence of metabolic changes within the muscle fibre (Allen et al. 2008b, a). The main culprit is inorganic phosphate (P_i), which accumulates during intense contractions as a by-product of ATP hydrolysis and ATP regeneration from phosphocreatine. Increased intracellular $[P_i]$ decreases both, cross-bridge force production and myofibrillar Ca^{2+}-sensitivity (Westerblad et al. 2002; Allen et al. 2008a). In addition, P_i reduces SR Ca^{2+} release, potentially by inhibition of the RyR1 and/or by reducing the availability of free Ca^{2+} in the SR by Ca^{2+}-P_i precipitation (Dahlstedt et al. 2003; Allen et al. 2008a, b). Another factor that might affect tetanic $[Ca^{2+}]_i$ and force during fatigue is the production of reactive oxygen species (ROS) by the mitochondria and the NADPH oxidase, which is enhanced during intense contractions (Powers and Jackson 2008; Sakellariou et al. 2013). Acute elevation of ROS increases myofibrillar Ca^{2+} sensitivity whereas prolonged elevated levels have the opposite effect (Andrade et al. 1998; Andrade et al. 2001; Cheng et al. 2015). In addition, excessive ROS production may alter SR Ca^{2+} release by oxidative modification of the RyR1 (Moopanar and Allen 2006; Westerblad and Allen 2011).

The ability of a muscle fibre to sustain contractile force during fatiguing stimulation varies considerably depending on its metabolic properties. Fibres with a large aerobic capacity (ie, type I fibres) are generally more fatigue-resistant than fibres that rely more on anaerobic metabolism (ie, type II fibres) (Allen et al. 2008a; Place et al. 2009; Zhang et al. 2006; Bruton et al. 2003). Reduced fatigue-resistance limits endurance exercise performance and is also a common symptom of neuromuscular disorders (Yamada et al. 2012; Petty et al. 1986; Drachman 1994; Allen et al. 2016). Endurance training stimulates mitochondrial biogenesis and increases the aerobic capacity and is therefore effective to counteract fatigue (Ivarsson et al. 2019; Bruton et al. 2010).

The force decline induced by fatigue is reversible but after fatiguing stimulation, fibres often exhibit prolonged periods of depressed force production, which is most pronounced when stimulated at a low stimulation frequency, ie, in the linear part of the force-$[Ca^{2+}]_i$ curve (Cheng et al. 2015). This type of delayed recovery from fatigue is referred to as prolonged low-frequency force depression (PLFFD) and related to a decrease in tetanic $[Ca^{2+}]_i$ and/or myofibrillar Ca^{2+}-sensitivity (Cheng et al. 2015). The recovery of tetanic $[Ca^{2+}]_i$ and force in a fatigued fibre can be accelerated by increasing the temperature whereas cooling has the opposite effect (Cheng et al. 2017).

1.1.4 The ryanodine receptor

In the early 1970s, Clara Franzini-Armstrong published ground-breaking electron micrographs of the junction between the t-tubules and the SR membrane in frog muscle fibres. The images showed large structures of unknown substance spanning from the t-tubule to the SR membrane that were named "feet" and that seemed to be involved in EC coupling (Franzini-Armstrong 1970). In the 1980s, a group of researchers localized the SR Ca^{2+} release channels in the terminal cisterna of the SR. Due to their high affinity to the plant alkaloid ryanodine, the channels were referred to as "ryanodine receptors" (Fleischer et al. 1985). Further studies identified the "feet" seen on the initial electrographs as the RyR1 (Inui et al. 1987). In the past 50 years, our understanding of the RyR1 function has grown considerably and recently, high-resolution 3D models obtained with electron cryo-microscopy have provided deeper insights in the structure of the channel and the mechanisms of EC coupling (Dulhunty 2006; Yan et al. 2015; Zalk et al. 2015).

The RyR1 is a mushroom-shaped protein complex with a stem-like transmembrane pore and a large cytoplasmic domain (the "cap of the mushroom") bridging the gap between the SR and the t-tubule (Van Petegem 2012; Hamilton and Serysheva 2009; Zalk and Marks 2017). With a total molecular mass of more than 2 MDa, the RyR1 is the largest known ion channel (Zalk and Marks 2017). The RyR1 is part of a macromolecular complex consisting of four identical RyR1 subunits with a molecular weight of approximately 550 kDa each, four FK506-binding proteins (FKBP12) and several associated proteins including calmodulin (CaM), protein kinases and protein phosphatases (Meissner 2017; Hamilton and Serysheva 2009). There are three isoforms of the RyR, which are encoded by separate genes (*RyR1*, *RyR2* and *RyR3*) and primarily expressed in skeletal muscle (RyR1), cardiac muscle (RyR2) and in smooth muscle, diaphragm and brain (RyR3) (Hamilton and Serysheva 2009). Mutations in *RyR1* and *RyR2* give rise to a number of severe skeletal and cardiac muscle diseases, including malignant hyperthermia, central core disease and cardiac arrhythmia (Dulhunty et al. 2018).

In skeletal muscle, EC coupling occurs via physical contact between the DHPR and the RyR1 and hence, DHPRs are organized in tetrads of four DHPRs aligned with the four subunits of the opposing RyR1 (Block et al. 1988; Paolini et al. 2004). Intriguingly, only every other RyR1 is coupled with a DHPR tetrad and the activation mechanism of the uncoupled RyR1s remains unclear (Dulhunty 2006).

1.1.5 Modulation of RyR1 function and SR Ca^{2+} leak

The cytosolic domain of the RyR1 is a scaffold for binding of molecules, ions, pharmaceutical drugs and regulatory proteins that modulate the channel function (Zalk et al. 2007). The RyR1 is a high-conductance gated ion channel and several compounds or posttranslational modifications alter the open probability and/or the mean open time of the channel, which alters SR Ca^{2+} release (Andersson and Marks 2010). In micromolar concentrations (up to ~10 µM), Ca^{2+} binds to different sites on the cytosolic domain and activates SR Ca^{2+} release in isolated RyR1s (Santulli et al. 2017; Van Petegem 2012; des

Georges et al. 2016). Ca^{2+} binding sites on the luminal side of the RyR1 suggest that the SR [Ca^{2+}] also affects channel function, however, these relationships are incompletely understood (Meissner 2017). Mg^{2+} has an inhibitory effect on isolated RyR1s by competitive binding to the cytosolic Ca^{2+} binding sites whereas ATP and other adenine nucleotides potentiate SR Ca^{2+} release (Meissner 2017; Lanner et al. 2010).

Of the many pharmaceutical compounds that modulate RyR1 function, ryanodine and caffeine have been studied in most detail. Ryanodine is a toxin, which, in micromolar concentrations, binds to a high-affinity site on the RyR1 and locks the channel in a half open state resulting in irreversible muscle contractures due to continuous SR Ca^{2+} leak (Santulli et al. 2017; Inui et al. 1987). At higher concentrations (in the millimolar range), ryanodine binds to low-affinity sites on the RyR1, which closes the channel completely and interrupts EC coupling (Meissner 2017). Caffeine increases the open probability and the mean open time of the RyR1 without interfering with other steps of EC coupling and is therefore used to experimentally deplete the SR from Ca^{2+} (Meissner 2017; Allen and Westerblad 1995; Rousseau et al. 1988).

The regulatory protein FKBP12 tightly associates with each of the RyR1 subunits and stabilizes the channel in its closed state (Ahern et al. 1994, 1997; Brillantes et al. 1994). Dissociation of FKBP12 by pharmacological intervention increases the open probability and the mean open time of the RyR1 resulting in so called “leaky” channels (Ahern et al. 1997). SR Ca^{2+} leak has been associated with muscle weakness in a number of pathologic conditions, including malignant hyperthermia, muscle dystrophy and arthritis (Lanner et al. 2012; Bellinger et al. 2009; Yamada et al. 2015). In age-related sarcopenia, oxidation and nitrosylation of the RyR1 resulted in FKBP12 dissociation and SR Ca^{2+} leak (Andersson et al. 2011). Similar destabilization of the RyR1 with subsequent Ca^{2+} leak was induced by intense exercise in humans and mice and speculated to be a mechanism underlying overtraining (Bellinger et al. 2008). In a healthy muscle fibre, resting $[Ca^{2+}]_i$ is tightly regulated and large, chronic elevations have deleterious effects on muscle function. Mild, acute increases, however, induce the expression of the peroxisome proliferator-activated receptor γ coactivator-1α1 (PGC-1α1), a regulator of mitochondrial biogenesis (Wright et al. 2007; Handschin et al. 2003; Arany 2008). In a recent study, mice who were given access to running wheels for three weeks showed decreased association of FKBP12 to the RyR1 and increased resting $[Ca^{2+}]_i$, as well as an increase in the expression of genes involved in mitochondrial biogenesis. Pharmacological destabilization of the RyR1-FKBP12 complex improved endurance exercise performance, suggesting that a mild Ca^{2+} leak is a trigger for muscular adaptations to exercise (Ivarsson et al. 2019). Place et al. have observed extensive remodelling of the RyR1 in muscle biopsies from untrained humans obtained 24 hours after a single bout of intensive sprint interval training (SIT) together with an upregulated expression of mitochondrial genes. Mechanistic experiments on isolated mouse muscle fibres confirmed that SIT induced a ROS-dependent SR Ca^{2+} leak and an increase in resting $[Ca^{2+}]_i$. Intriguingly, endurance athletes had no fragmentation of the RyR1 after performing the same SIT exercise indicating that endurance training has a protective effect on RyR1 modifications (Place et al. 2015).

1.1.6 Muscle weakness in amyotrophic lateral sclerosis

Muscle weakness and premature fatigue are symptoms of a wide range of neuromuscular disorders, which impede activities of daily living and cause disability. The following chapter will discuss the origin of muscle weakness in amyotrophic lateral sclerosis (ALS), a neurological disorder characterized by the gradual degeneration of motor neurons (Hardiman et al. 2017; Julien and Kriz 2006). ALS is rare, affecting approximately 6 adults in 100,000 (Talbott et al. 2016). The progressive loss of motor neurons causes muscle cramps, spasticity, atrophy, muscle weakness and ultimately respiratory failure (Hardiman et al. 2017). In most cases, ALS is diagnosed in the fifth or sixth decade of life and progresses rapidly leading to premature death within 2-3 years although individual cases of long-term survival exist (Al-Chalabi and Hardiman 2013; Boyer et al. 2013; Chio et al. 2009; Pupillo et al. 2014).

ALS is a heterogeneous disease occurring sporadic in most cases. However, around 10% of the patients have a family history of ALS and of these, ~20% are associated with mutations in the *SOD1* gene encoding the antioxidant enzyme superoxide dismutase 1 (SOD1) (Al-Chalabi and Hardiman 2013; Gurney et al. 1994; Julien and Kriz 2006). To date, more than 180 different mutations of *SOD1* have been identified in patients with familial ALS (Alrafiah 2018). One of the histologic hallmarks of ALS are protein aggregates in the motor neurons and accumulation of misfolded SOD1 protein has been observed in patients with *SOD1* mutations (Hardiman et al. 2017; Julien and Kriz 2006).

As a model of ALS, transgenic mice harbouring human SOD1 mutations have been generated. One of the most commonly used animal models in this respect is a transgenic mouse strain overexpressing SOD1 protein with a point mutation in amino acid 93 (glycine substituted with alanine) ($SOD1^{G93A}$) (Julien and Kriz 2006). The $SOD1^{G93A}$ mice have a high rate of SOD1 synthesis (~40 times that of wild type mice) and develop an adult-onset motor neuron disease similar to ALS with motor neuron degeneration, muscle weakness, paralysis and premature death at ~150 days of age (Atkin et al. 2005; Gurney et al. 1994; Hardiman et al. 2017; Valentine et al. 2005).

The treatment options for patients with ALS are limited and a major barrier for the development of therapies is the lack of understanding of the underlying molecular mechanisms. Muscle weakness is a cardinal symptom of ALS with a large impact on the patients' quality of life. Muscle weakness is considered a consequence of reduced muscle mass (due to muscle fibre atrophy and/or loss of muscle fibres) caused by denervation and this was confirmed by Hegedus et al., who demonstrated that reduced contractile force in whole *tibialis anterior* muscles of $SOD1^{G93A}$ mice was attributed to selective atrophy of type IIB muscle fibres (Gurney et al. 1994; Hegedus et al. 2008). However, more recent studies suggest that muscle intrinsic defects (ie, a reduction in the specific contractile force of individual muscle fibres) contribute to muscle weakness in ALS and one theory is that the expression of mutated SOD1 protein leads to cellular toxicity that impairs contractile function (Dobrowolny et al. 2008; Wong and Martin 2010). Beqollari et al. found muscle intrinsic defects with reduced tetanic $[Ca^{2+}]_i$ and impaired EC coupling and another study observed increased resting $[Ca^{2+}]_i$ and decreased expression of SERCA1 in enzymatically dissociated

flexor digitorum brevis (FDB) muscle fibres from $SOD1^{G93A}$ mice (Beqollari et al. 2016; Chin et al. 2014)

1.1.7 Effect of skeletal muscle adaptations on thermoregulation

Apart from its force-generating tasks, skeletal muscle is involved in a range of metabolic and regulatory processes, including thermoregulation (Pedersen and Febbraio 2012). To maintain core temperature in cold environments, skeletal muscle contributes to thermoregulation by shivering and non-shivering thermogenesis. This section discusses how the lack of the structural protein α-actinin-3 (ACTN3) in skeletal muscle may induce alterations in Ca^{2+} handling that are advantageous for survival in cold environments.

α-actinins are a family of actin-binding proteins that cross-link actin to intracellular structures in different cell types (Blanchard et al. 1989). The skeletal muscle isoforms α-actinin-2 (ACTN2) and ACTN3 are major components of the Z-line anchoring the actin filaments of adjoining sarcomeres (Hogarth et al. 2017). Furthermore, ACTN2 and ACTN3 interact with structural proteins, metabolic enzymes, and signalling proteins involved in Ca^{2+} handling suggesting functions beyond their structural role (Quinlan et al. 2010). ACTN2 is ubiquitously expressed in all muscle fibre types, whereas the expression of ACTN3 is limited to fast, glycolytic fibres (Head et al. 2015). The absence of ACTN3 was first discovered in skeletal muscles of patients with muscular dystrophy in the late 1990s and gained attention as a potential cause for the disease (North and Beggs 1996). Homozygosity for a nonsense-mutation in the *ACTN3* gene with a premature stop codon instead of an arginine at residue 577 (577XX) was identified as the underlying cause of ACTN3 deficiency, however, the putative role for muscle dystrophy was put into perspective when the 577XX genotype was also found in healthy siblings of the affected patients (MacArthur and North 2004). In fact, ACTN3 deficiency turned out to be surprisingly frequent in the general population (~16-18%) and more than a billion people worldwide are estimated to carry the 577XX mutation (North et al. 1999; Head et al. 2015; MacArthur et al. 2007). The expression of ACTN2 is upregulated in ACTN3 deficiency, which may explain the absence of a pathologic phenotype (Mills et al. 2001; MacArthur et al. 2007). The observation that the 577XX genotype is underrepresented among groups of elite power and sprint athletes led to the hypothesis that ACTN3 has a functional role for force generation and muscle power, which is supported by evidence of reduced muscle strength in an ACTN3 knockout ($ACTN3^{-/-}$) mice (Yang et al. 2003; Niemi and Majamaa 2005; Papadimitriou et al. 2008; Druzhevskaya et al. 2008; Eynon et al. 2009; MacArthur et al. 2008). In contrast, the frequency of the 577XX genotype is overrepresented in female elite endurance athletes, suggesting potential advantages of ACTN3 deficiency for endurance exercise (Yang et al. 2003). Indeed, $ACTN3^{-/-}$ mice exhibited better endurance capacity than their wild type littermates presumably due increased oxidative enzyme activity and mitochondrial density and hence higher muscle oxidative capacity (MacArthur et al. 2007).

There is a geographic variation in the prevalence of the 577XX genotype with a higher frequency in places where the temperature is low and food sources are scarce suggesting an

evolutionary advantage in such environments (Friedlander et al. 2013; Houweling et al. 2018; MacArthur et al. 2007). Recent experiments on enzymatically dissected FDB muscle fibres from *ACTN3*$^{-/-}$ mice provided evidence for altered intracellular Ca^{2+} handling in ACTN3 deficiency with increased SR Ca^{2+} leak and reuptake (Head et al. 2015). Wild type mice that are acclimatized to the cold exhibit similar increases in Ca^{2+} cycling (Bruton et al. 2010). The reuptake of Ca^{2+} by the SERCA pump is driven by the hydrolysis of ATP and thus generates heat. As such, increased Ca^{2+} cycling could contribute to non-shivering thermogenesis. It has been hypothesized that the 577XX allele has undergone positive selection during recent evolution when humans inhabited cold areas, however, whether increased skeletal muscle Ca^{2+} cycling is also implicated in humans remains to be established (MacArthur et al. 2007).

1.1.8 Effect of skeletal muscle adaptations on depressive disorder

Depression is a mental disorder with negative impacts on everyday activities, social interactions and quality of life (Kennedy et al. 2001). With more than 300 million people affected, depression is the leading cause of disability in the world and a major burden for the health-care system (Ferrari et al. 2013; WHO 2018; Gustavsson et al. 2011). The molecular mechanisms underlying depression are not fully understood, but in the last decade an increasing number of studies have reported elevated inflammatory markers in the plasma of depressed patients, linking stress and abnormal immune function to the pathogenesis (Notarangelo et al. 2018). One of the potential key players is the enzyme indoleamine 2,3-dioxygenase (IDO), which is rapidly activated by inflammatory markers and accelerates the conversion of the essential amino acid tryptophan to kynureine (KYN) (Notarangelo et al. 2018). The KYN pathway generates several neuroactive metabolites that are strongly related to mental disorders. The degradation of KYN follows one of two possible branches: KYN is either converted to 3-hydroxykynurenine (3HK) and quinolinic acid (QUIN) or alternatively to kynurenic acid (KYNA), a reaction catalysed by kynurenine amino transferases (KATs) (Cervenka et al. 2017) (Fig. 2). QUIN is neurotoxic and acts as a N-methyl-D-aspartate (NDMA) receptor agonist in the brain, whereas KYNA is a NDMA antagonist with neuroprotective properties (Erhardt et al. 2017). KYNA cannot pass the blood brain barrier and hence, brain levels of KYNA are solely determined by local production (Fukui et al. 1991; Gal and Sherman 1980). In contrast, KYN passes readily from the blood to the brain and peripheral accumulation of KYN may therefore affect brain levels (Fukui et al. 1991). Increased concentrations of QUIN in the brain, as well as imbalances of the KYN metabolites in the periphery, have been associated with depression (Claes et al. 2011; Erhardt et al. 2017; Muller and Schwarz 2007).

Traditionally, patients with depression are managed with a combination of drugs and psychotherapy. More recently, physical exercise has been added to the treatment guidelines as complementary or alternative therapy (Lawlor and Hopker 2001; Chalder et al. 2012; Guidelines 2010). While the beneficial effects of exercise on general well-being are commonly recognized, the underlying molecular mechanisms remain poorly understood (Mead et al. 2009). The recent discovery of KATs in skeletal muscle has added new evidence

for the crosstalk between muscle and brain with potential implications for depressive disorders: Endurance exercise induced the expression of skeletal muscle KATs in mice via activation of the PGC-1α1/PPARα/δ pathway. Increased expression of skeletal muscle KATs resulted in a shift of the peripheral KYN metabolism towards the production of KYNA thereby reducing the accumulation of KYN and its neurotoxic metabolites in the brain. Hence, animals with increased skeletal muscle KAT expression were resilient to depression induced by chronic mild stress or by administration of KYN (Agudelo et al. 2014). Sedentary humans who participated in a three-week endurance exercise program had also increased skeletal muscle KAT gene expression, however, adaptations of KAT protein levels and plasma kynurenine metabolites with exercise have not yet been established in humans (Agudelo et al. 2014).

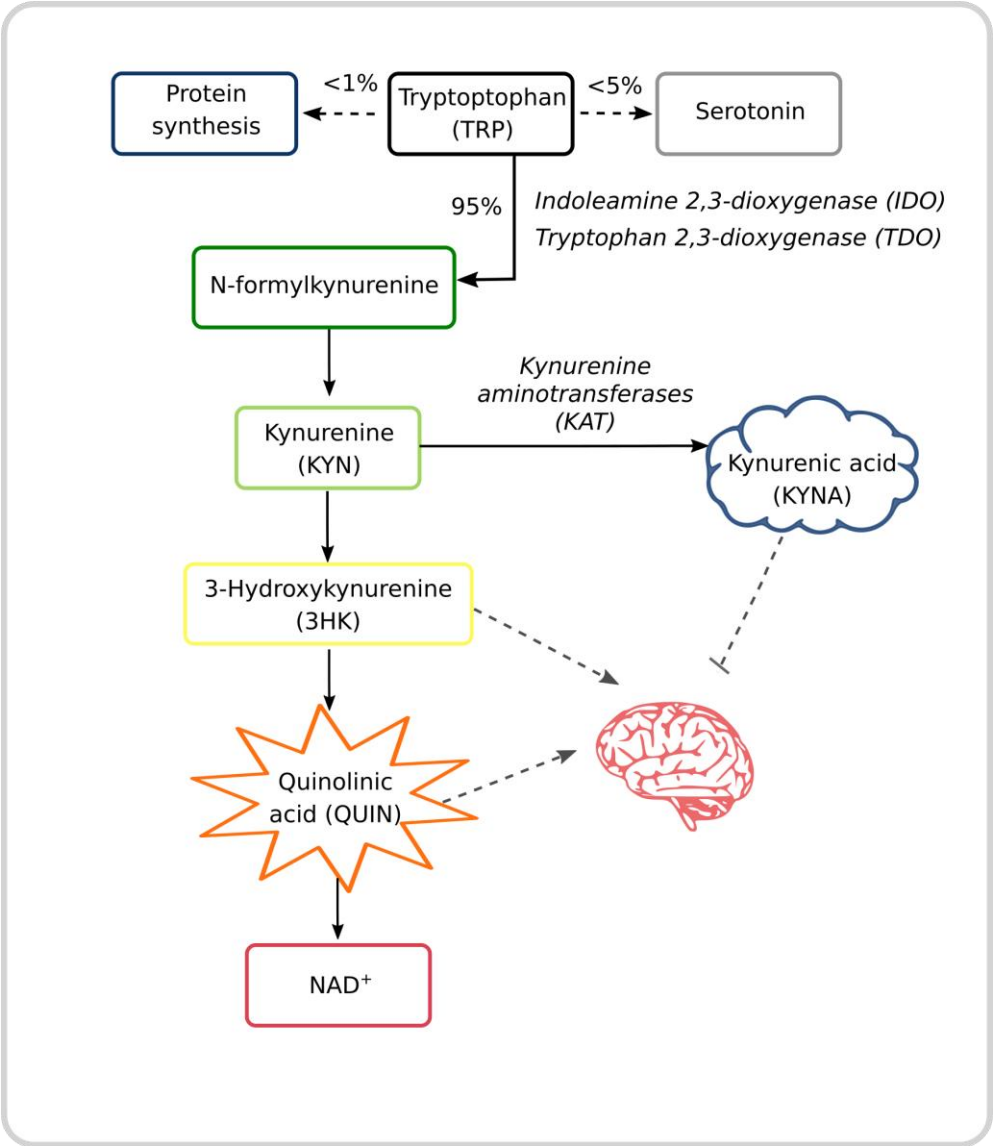

Figure 2. Simplified representation of the tryptophan degradation pathway. Kynurenine (KYN) is either converted to the neuroprotective kynurenic acid (KYNA) via kynurenine aminotransferases (KATs), or to 3-hydrokynurenine (3HK) and the neurotoxic quinolinic acid (QUIN). Note that while KYN, 3 HK and QUIN can pass from the periphery to the brain, KYNA cannot cross the blood brain barrier.

2 AIMS

Skeletal muscle is a plastic tissue that responds to external stimuli including different types of exercise, physical inactivity and prolonged disuse. Molecular adaptations at the muscle fibre level include changes in cross-sectional area, altered intracellular Ca^{2+} handling, changes in the contractile properties of the myofibrils and altered expression of proteins and enzymes. All of these changes can locally affect force production and/or fatigue-resistance with implications for everyday activities, locomotion and athletic performance. As skeletal muscle is involved in a number of metabolic pathways and regulatory functions, molecular adaptations may moreover have systemic effects. Increased intracellular Ca^{2+} handling, for example, increases the activity of SERCA, which requires ATP and generates heat, hence affecting whole-body metabolism and thermoregulation and altered expression of specific proteins and enzymes can change the metabolic properties of the muscle fibre with effects on energy homeostasis and cross-talk between muscles and other organs. The overall aim of this thesis was to study molecular adaptations of skeletal muscle and their local and systemic effects in physiological and pathological conditions.

The specific aims were:

- To study local effects of SIT on RyR1 integrity and on neuromuscular function in humans (Paper I).
- To investigate the contribution of local changes in Ca^{2+} handling and altered contractile properties for muscle weakness in a mouse model of ALS (Paper II).
- To examine the effect of ACTN3 deficiency on skeletal muscle Ca^{2+} handling and on thermogenesis in humans (Paper III)
- To explore the effect of different types of exercise on peripheral KYN metabolism in humans with implications on muscle-brain cross-talk (Paper IV).

3 METHODS

A brief overview of the main methods used in this thesis is provided in the following sections. A more detailed description of all the experimental procedures is available in the respective papers.

3.1 EXERCISE PROTOCOLS

The SIT intervention for paper I consisted of a three-week training program performed on a cycle ergometer including a total of 9 SIT sessions. Each SIT session started with a 5 min warm-up at ~100 W, followed by 4-6 30 s all-out cycling bouts (Wingate tests) with 4 min rest in between. The 30 s sprints were performed against a resistance of 0.7 Nm/kg body weight. After the last sprint, subjects cooled down according to their individual preference. Maximal cycling power (W_{max}) before and after the three-week intervention was assessed using an incremental test to failure on a cycle ergometer. The test started with 3 min of cycling at 50 W after which the load was increased by 5 W every 10 s. Subjects were told to pedal at a cadence of 60 rpm and the test was continued until the required cadence could no longer be maintained.

3.2 BLOOD SAMPLES

For paper IV, venous blood samples were collected in EDTA-treated vacutainers and separated by centrifugation for 15 min at 3,000 rpm and 4°C. Blood plasma was stored at -80°C until analysis.

3.3 MUSCLE BIOPSIES

Vastus lateralis muscle biopsies were collected from human subjects for papers I, III an IV. The biopsy site was cleaned with alcohol and locally anesthetized and a small skin cut was made using a scalpel tip. A disposable biopsy needle was inserted perpendicular to the muscle fibres until the fascia was pierced and biopsies of 15-30 mg were collected using an automatic biopsy device. The biopsies were cleaned, frozen in liquid nitrogen and stored at -80°C until analysis. The skin cut was cleaned and closed with wound closure strips.

3.4 WESTERN BLOTTING

Western blots were performed for all four papers. Frozen muscle tissue was homogenized in lysis buffer and lysates were centrifuged (papers I, II and IV) at 700 g for 10 min at 4°C. After quantifying the protein content of the homogenates, samples were diluted 1:1 in loading buffer and heated for 5 min at 95°C. 5-10 µg of protein were loaded on precast gels and separated for 60-90 min at 150 V. Proteins were then wet transferred to polyvinylidine fluoride membranes during 180 min at 100 V. After blocking, membranes were incubated in primary antibody at 4°C over night. After washing and incubating with secondary antibody, bands were visualized by infrared fluorescence. Band densities were normalized either to a reference protein or to total protein content of each lane.

3.5 GENE EXPRESSION ANALYSIS

In papers II and IV, total RNA was isolated from frozen mouse tissue using TRIzol. After treatment with DNAse, total RNA concentration was assessed and reverse transcription was performed. Quantitative real-time PCR was performed using SYBR Green and a Real-Time PCR system thermal cycler. Gene expression analysis was performed according to the ΔΔCt method.

3.6 FIBRE TYPING

The myosin heavy chain isoforms of homogenized muscle samples were separated by electrophoresis on specifically casted separation gels for papers II and III. ~100 ng of protein were loaded in each lane of the gel and electrophoresis was run for 22 h at 4°C. The gels were then stained with a silver staining kit and band densities were quantified using ImageJ software.

3.7 RYR1 IMMUNOPRECIPITATION

For paper III, the association of FKBP12 with RyR1 was tested using immunoprecipiation of the RyR1. For the immunoprecipitation, supramagnetic, G-protein coupled beads were bound to the RyR1 antibody according to the manufacturer's instructions. The antibody-bead complex was added to muscle homogenates and incubated over night at 4ºC under gentle rotation. Samples were then washed four times with lysis buffer. To separate the RyR1 complex from the beads, Laemmli buffer with 5% beta-mercaptoethanol was added and samples were heated for 5 min at 95°C. The beads were then removed from the solution using a magnetic rack. Proteins were then separated by electrophoresis on precast gels, transferred to polyvinylidine fluoride membranes and incubated in primary and secondary antibodies as described in the western blotting section.

3.8 FORCE MEASUREMENTS ON ISOLATED WHOLE MUSCLES

For paper II, whole muscle force measurements were performed on FDB muscles. FDBs were isolated from the hind limbs of the sacrificed mice. Slings made from surgical suture were tied to the distal and proximal tendons of the FDBs and muscles were then mounted to a force transducer in a stimulation chamber. The chamber was filled with Tyrode solution and bubbled with 95% O_2 and 5% CO_2 at room temperature. Muscles were carefully stretched to optimal length and contractile force was controlled with 70 Hz stimulations of 350 ms duration. Contractile force was assessed by tetanic stimulation with gradually increasing stimulation frequency from 10 to 150 Hz. To avoid fatigue, stimulations were intermitted by one-minute resting periods. After assessment of contractile force, muscles were blotted dry, weighed and frozen in liquid nitrogen.

3.9 FORCE AND $[Ca^{2+}]_i$ MEASUREMENTS IN ISOLATED SINGLE MUSCLE FIBRES

For paper II, real-time measurements of contractile properties and $[Ca^{2+}]_i$ were performed in living single muscle fibres isolated from mouse FDB muscles. Under a microscope, an intact living single muscle fibre with tendons attached on both sides was dissected manually. Small clips made of aluminium were attached to each of the tendons and the fibre was then mounted onto two hooks connected to a force-transducer in a stimulation chamber. The stimulation chamber was continuously superfused with Typrode solution at 23°C. To measure $[Ca^{2+}{}_i]$, the fibre was microinjected with the fluorescent indicator indo-1. The dye was then excited at 346 nm with a Xenon lamp and light emitted at 405 nm and 485 nm was captured by two photomultiplier tubes. The fluorescence signals was translated to $[Ca^{2+}]_i$ using intracellular calibration. To measure contractile force, fibres were gently stretched to optimal length and subsequently stimulated with tetani of 10 – 150 Hz. Fatigue was induced by intermittent (70 Hz, 350 ms at 2 s intervals) tetanic stimulation repeated for 150 contractions.

4 RESULTS AND DISCUSSION

4.1 EFFECT OF SPRINT INTERVAL TRAINING ON RYR1 MODIFICATIONS (PAPER I)

SIT is a popular and time-efficient training form alternating short bouts of maximal or supramaximal exercise with periods of rest. The molecular mechanisms that mediate training adaptations in response to SIT are incompletely understood but a recent study has observed fragmentation and reduced abundance of the RyR1 in muscle biopsies from untrained human subjects 24 h after exposure to a single SIT session. Electrical stimulation of isolated mouse muscle fibres with a SIT-mimicking protocol caused a SR Ca^{2+} leak and a subsequent increase in resting $[Ca^{2+}]_i$ (Place et al. 2015). Together, this has led to the hypothesis that SIT induces alterations in the RyR1, which result in SR Ca^{2+} leak and an increase in resting $[Ca^{2+}]_i$. A prolonged, mild increase in resting $[Ca^{2+}]_i$ has been shown to stimulate mitochondrial biogenesis and improve fatigue resistance (Bruton et al. 2010; Ivarsson et al. 2019). Intriguingly, endurance trained subjects who performed the same SIT session as the untrained subjects did not show alterations of the RyR1, suggesting that training protects from RyR1 modifications (Place et al. 2015). The aim of the present study was to expose untrained subjects to a three-week SIT program and measure cycling performance, RyR1 modifications and recovery of contractile force at the start and end of the training period. We hypothesized that repeated exposure to SIT would induce some protection against RyR1 modifications, improve cycling performance and accelerate recovery of neuromuscular function. Eight recreationally active male subjects participated in the three-week SIT program including a total of 9 SIT sessions, each consisting of 4-6 30 s sprints with 4 min rest in between.

Throughout the intervention, the subjects performed 47 all-out sprints, accumulating to a total sprint time of 23.5 min. This low volume but high-intensity exercise regime improved cycling performance in all participants. The total work performed during the last SIT session was 7.4% higher than during the first SIT session and a similar improvement was observed in the W_{max} reached during an incremental cycling. Other studies using similar SIT protocols reported comparable improvements (Weston et al. 2014). Of note, one SIT session includes a warm-up, resting periods between the sprints and a cool-down and takes approximately 35 min. Nevertheless, SIT seems a time-efficient method to improve exercise performance in recreationally active people.

In the previously mentioned study, SIT induced modifications of the RyR1 in untrained but not in trained people 24 h after exercise. Here, we analyzed biopsies collected 24 h after the first (ie, untrained state) and the last (ie, trained state) SIT session. Expression of the full-size RyR1 protein 24 h post SIT was lower in the untrained (Fig. 3A) than in the trained (Fig. 3B) state but the reduction in the untrained state was not as marked as previously reported (Place et al. 2015). The variability between subjects was rather large and a bigger sample size would have been required for clearer results. Two subjects had a reduction of the RyR1 in the trained state and hence, the training did not induce protective effects in these individuals. Of note, both subjects had a markedly larger improvement in the total work performed during

SIT than the rest of the subjects. 72 h after SIT, the RyR1 expression was recovered in the untrained and trained state. Despite physical interaction between the DHPR and the RyR1, the protein expression of the DHPR was not affected by SIT at any time point.

Neuromuscular function was assessed before and immediately, 1 h, 24 h and 72 h after the first and the last SIT session. We hypothesized that three weeks of SIT would improve neuromuscular recovery after SIT. Force during maximal voluntary contraction (MVC) was depressed immediately (~40% decrease) and 1 h (~25% decrease) after SIT and recovered 24 h after exercise with no difference between the untrained and trained state (Fig. 3C). Contractile force induced by electrical stimulation at low (10 Hz; PS10) and high (100 Hz; PS100) stimulation frequencies was also decreased immediately and 1 h after SIT and this decrease was not altered by training (Fig. 3D). Of note, force depression was similar after SIT in the untrained and trained state but the total work performed during SIT was higher in the trained state so in this regard, training had some positive effect on recovery of neuromuscular function.

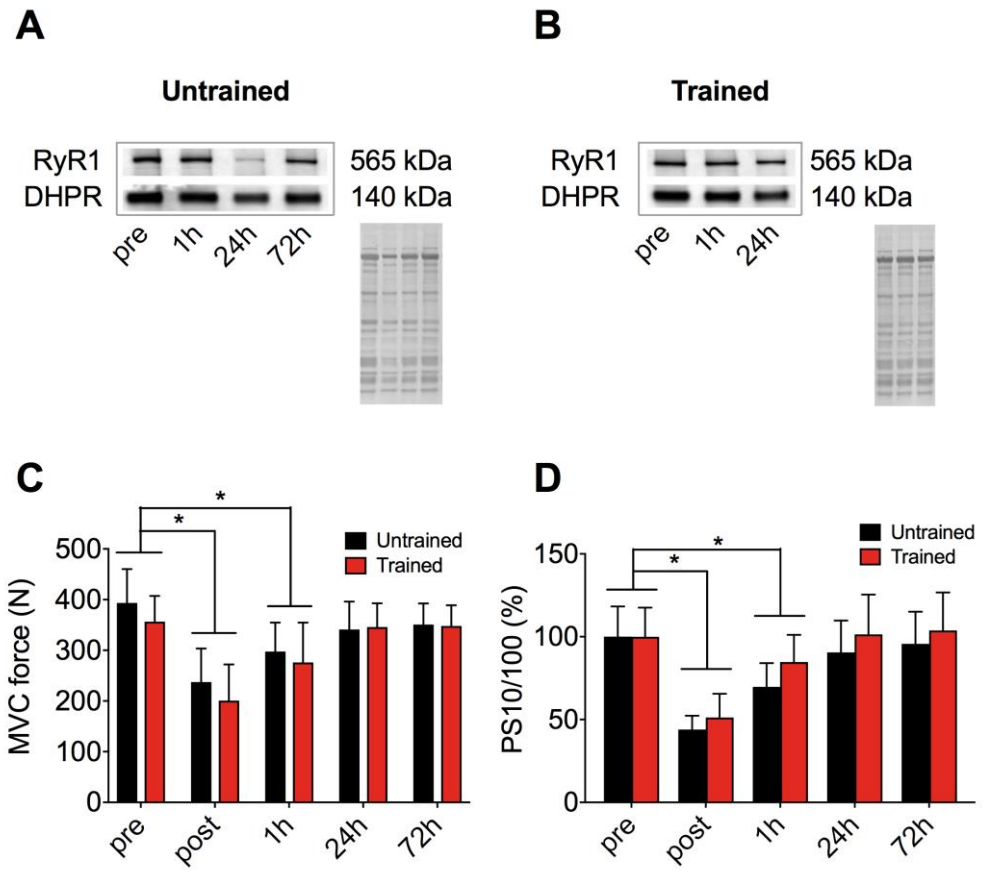

Figure 3. SIT-induced changes in contractile force and in full-length RyR1 protein expression. Representative RyR1 and DHPR bands from Western blots before (pre) and 1 h, 24 h and 72 h after a SIT session in the untrained **(A)** and trained state **(B)**. **(C)** Maximal voluntary contraction (MVC) force is reduced immediately and 1 h after a SIT session in the untrained and trained states. **(D)** Force depression after SIT is more marked at low than at high stimulation frequencies, leading to a decrease in the PS10/100 ratio.

To test whether force depression after SIT was caused by a reduced neural drive, we measured the voluntary activation level (VAL), which is an index of central fatigue. VAL was not affected by SIT at any time point in the untrained and trained state, suggesting that the reduced contractile force is of intramuscular origin. A potential explanation for depressed MVC and electrically induced forces is a decrease in sarcolemmal excitability, which can be measured with electromyography (EMG). The M-wave amplitude on the EMG was unaltered by SIT in the untrained and trained state indicating intact sarcolemmal excitability. The depression in electrically evoked contractile force after SIT was more pronounced at low than at high stimulation frequencies, hence, on the steep part of the force-$[Ca^{2+}]_i$ relationship. This suggests either a reduced Ca^{2+} release from the SR or decreased myofibrillar Ca^{2+} sensitivity as underlying cause of force depression. It has been hypothesized that modification of the RyR1 leads to defective SR Ca^{2+} release affecting contractile force. However, in our study, MVC and electrically evoked forces had recovered 24 h after SIT in the untrained state when the RyR1 expression was decreased. In the trained state, force depression was similar to the untrained state despite some protection of the RyR1. Our data is in line with our previous study, where force was recovered 24 h post SIT in untrained subjects while the RyR1 was fragmented and where trained subjects showed force depression without RyR1 modification (Place et al. 2015). Hence, we have to exclude a causative relationship between decreased RyR1 protein expression and reduced contractile force.

In summary, three weeks of SIT induced training adaptations that resulted in improved cycling performance and provided some protection of RyR1 modifications. Recovery of neuromuscular function was similar before and after training and was not associated with RyR1 modifications.

4.2 ORIGIN OF MUSCLE WEAKNESS IN ALS (PAPER II)

Progressive muscle weakness is the primary symptom of ALS patients and the main contributor to premature mortality due to respiratory failure. Denervation and subsequent muscle atrophy is the most obvious cause for decreased contractile force. However, recent evidence suggests that muscle fibre intrinsic defects, ie, a reduction in the specific force of individual muscle fibres, contribute to the development of muscle weakness. The treatment options for patients with ALS are limited and understanding the underlying causes of reduced muscle strength is essential for the development of therapies. The aim of the present study was therefore to investigate whether muscle weakness in ALS is caused solely by muscle atrophy or whether muscle fibre intrinsic factors, such as altered Ca^{2+} handling or altered contractile properties contribute to reduced contractile force.

Experiments were performed on a mouse model of ALS overexpressing a human mutation of the *SOD1* gene ($SOD1^{G93A}$). The mice were divided into two age groups: young, asymptomatic mice (P50; 50 days of age) and old, symptomatic or terminal mice (P125-150; 125-150 days of age). While there were no obvious phenotypic differences between the early stage $SOD1^{G93A}$ mice and their wild type littermates, late stage $SOD1^{G93A}$ mice showed marked hindlimb muscle wasting and significant weight loss. Motor neuron retrograde

labelling revealed a reduced number of motor neurons innervating the FDB muscle at the spinal level in symptomatic SOD1^{G93A} mice, confirming the pattern of progressive motor neuron degeneration (Fig. 4A). In addition, the expression of several genes involved autophagy (*Bnip3*), apoptosis (*Casp3* and *Casp7*) and proteasome proteolysis (*Murf1* and *Mul1*) was increased in SOD1^{G93A} mice suggesting activation of atrophy signalling.

In conjunction with this, isolated FDB muscles of late stage SOD1^{G93A} mice showed decreased muscle weight and reduced absolute force. However, when normalized to muscle weight, force was not different between SOD1^{G93A} mice and their wild type littermates. Moreover, the force-frequency relationship as well as the muscle fibre distribution was similar in both groups. In line with previous studies, these results suggest that the observed reduction in muscle force is primarily a consequence of progressive denervation and loss of muscle mass (Atkin et al. 2005; Mahoney et al. 2006; Hegedus et al. 2008). In contrast, studies on mice with muscle-specific overexpression of mutated SOD1 found reduced specific force in isolated *extensor digitorum longus* and *soleus* muscles (Dobrowolny et al. 2008), suggesting a deficiency in the contractile machinery in addition to muscle atrophy. This diverging finding may be explained by the use of different animal models and the different muscles studied. Yet, these discrepancies underscore the importance of carefully investigating the role of intrinsic defects in muscle function linked to ALS.

Although normalized force in whole FDBs was unaltered, estimation of specific force is complicated by the architecture of the FDB with multiple distal tendons and nonparallel arrangement of muscle fibres. Thus, the contractile properties were further investigated in mechanically dissected intact single muscle fibres, allowing for direct measurement of force, $[Ca^{2+}]_i$ and cross-sectional area. To test for potential defects in EC coupling, the membrane excitability of single muscle fibres was assessed by increasing the stimulation voltage until a twitch was elicited. There was no difference in membrane excitability between SOD1^{G93A} mice and wild types in both age groups and moreover, single fibres of late stage SOD1^{G93A} mice exhibited normal tetanic $[Ca^{2+}]_i$, (Fig. 4B) preserved tetanic force (Fig. 4C) and no changes in resting $[Ca^{2+}]_i$. Thus, there were no signs of defective EC coupling or impaired Ca^{2+} handling in the SOD1^{G93A} muscle fibres. In contrast, previous studies have reported defective impaired membrane excitability (Beqollari et al. 2016), decreased Ca^{2+} release from the SR (Beqollari et al. 2016) and increased resting $[Ca^{2+}]_i$ (Chin et al. 2014) in enzymatically dissociated muscle fibres from SOD1^{G93A} mice. These contradictory findings may potentially be explained by the use of mechanically dissected versus enzymatically dissociated muscle fibres. During mechanical dissection, muscle fibres are electrically stimulated and contracting fibres are dissected for the experiment. This may introduce some bias where strong, contracting muscle fibres are chosen over weak, non-contracting fibres and hence defective EC coupling or Ca^{2+} handling cannot be completely ruled out. However, the same risk of introducing bias exists for enzymatically isolated fibres that were used in the previous studies as only fibres that survive the dissociation process can be used for experiments. Nevertheless, the results of our single fibre experiments suggest that the surviving fibres in SOD1^{G93A} mice have intact contractile properties and this is consistent with muscle fibres from ALS patients,

which show a decrease in total muscle force without changes in specific force of single muscle fibres (Krivickas et al. 2002).

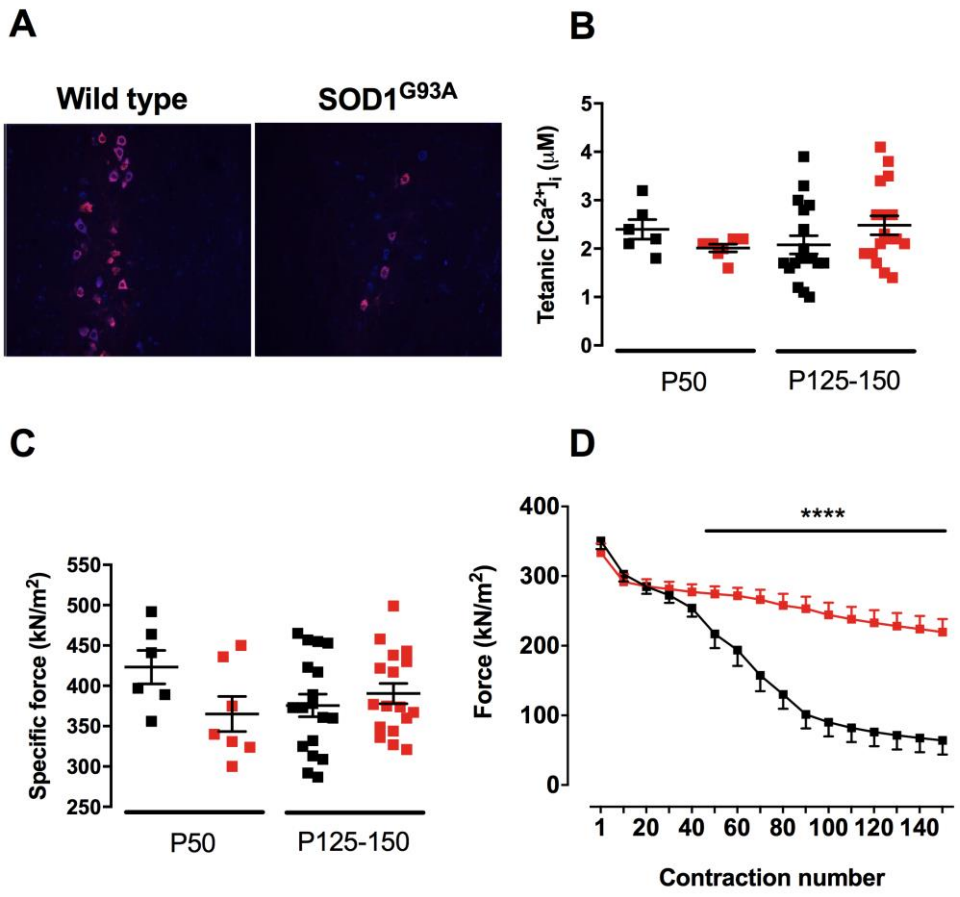

Figure. 4. FDB denervation but intact single fibre properties in SOD1^{G93A} mice. (A) Loss of motor neurons innervating the FDB muscle at the spinal level in SOD1^{G93A} mice. Single muscle fibres from SOD1^{G93A} mice (red) and wild type littermates (black) display preserved tetanic $[Ca^{2+}]_i$ **(B)** and specific force **(C)**. **(D)** Single fibres from late stage SOD1^{G93A} mice (red) have increased fatigue-resistance compared to the wild type littermates (black).

The fatigue-resistance of a single muscle fibre is primarily determined by its metabolic properties. To determine whether muscle fibres of SOD1^{G93A} mice exhibit defects in energy metabolism, fibres were exposed to fatiguing stimulation consisting of 150 repeated submaximal tetanic contractions at 70 Hz. At 50 days of age, tetanic $[Ca^{2+}]_i$ and force were similar in SOD1^{G93A} and wild type mice. Intriguingly, in the late stage, muscle fibres of SOD1^{G93A} mice exhibited increased fatigue-resistance compared to the age-matched controls (Fig. 4D) with preserved tetanic $[Ca^{2+}]_i$ and force during fatiguing stimulation. In addition, the rise in resting $[Ca^{2+}]_i$ during fatigue was significantly lower in late stage SOD1^{G93A} mice than in the wild type littermates indicating improved SR Ca^{2+} reuptake. These results seem counterintuitive as patients with ALS typically experience muscle fatigue (Sharma et al. 1995). However, the increased perception of muscle fatigue may be attributed to the impaired ability to fully activate the muscle due to motor neuron loss. Hence, in order to perform a given task, a greater volitional effort is required, which is perceived as fatigue. In support of

the presented results, previous studies have reported increased fatigue-resistance in whole muscles of symptomatic SOD1^{G93A} mice and in another ALS mouse model (Derave et al. 2003; Joyce et al. 2015), ascribed to the loss of fast-twitch muscle fibres and an increased proportion of slow-twitch fibres. In our study, fibre type distribution was unaltered suggesting that increased fatigue-resistance in SOD1^{G93A} mice is caused by intrinsic metabolic adaptations rather than fibre type shift.

To test for increased oxidative capacity, gene and protein expression of mitochondrial markers were analysed using western blotting and real-time qPCR. The expression of genes involved in mitochondrial biogenesis (*PGC-1α1, Nrf1* and *TFAM*) was upregulated in early and late stage SOD1^{G93A} mice compared to the wild type littermates. In agreement with this, protein expression of the mitochondrial electron transport chain complexes II, IV and V were increased in late stage SOD1^{G93A} mice. Intriguingly, there was no difference in the expression of the mitochondrial marker VDAC and in citrate synthase activity. Late stage SOD1^{G93A} mice had a marked increase in myoglobin protein content, which is typically associated with an increased oxidative capacity and endurance training (Ordway and Garry 2004; Schiaffino and Reggiani 2011; Takakura et al. 2015).

In summary, the present study provides evidence for intact specific force and increased fatigue-resistance in surviving muscle fibres of symptomatic SOD1^{G93A} mice confirming that muscle weakness is caused by denervation and atrophy and not by muscle fibre intrinsic defects. Our data suggests that surviving muscle fibres maintain their ability to adapt, which may be of importance for training recommendations and pharmaceutical interventions for ALS patients. All experiments in this study were performed on a mouse model carrying a specific mutation of the *SOD1* gene, representing a subgroup of ALS patients with a specific congenital form of ALS rather than the more common sporadic form of ALS. Moreover, SOD1^{G93A} mice have a massively increased rate of SOD1 synthesis in order to develop the pathologic phenotype in the lifespan of a mouse, which should be taken into consideration when drawing conclusions for ALS patients (Julien and Kriz 2006). Yet, the SOD1^{G93A} mouse is an established animal model of ALS used to investigate disease mechanisms.

4.3 COLD-RESILIENCE IN ACTN3 DEFICIENCY (PAPER III)

More than a billion people worldwide are homozygous for a null polymorphism of the *ACTN3* gene (577XX) resulting in complete deficiency of the skeletal muscle structural protein ACTN3 (North et al. 1999). The absence of ACTN3 is not associated with a pathologic phenotype, however, several studies have suggested altered muscle function and decreased muscle power (MacArthur et al. 2008). Intriguingly, there is a geographic variation in the frequency of the 577XX genotype with a higher prevalence in places with low temperature (Friedlander et al. 2013). Genetic studies have led to the hypothesis that the 577XX allele has undergone strong positive selection during recent evolution, when humans inhabited colder areas (MacArthur et al. 2007). A recent study on *ACTN3*$^{-/-}$ mice has provided a potential explanation for a survival advantage in cold climate: enzymatically dissected FDB muscle fibres of *ACTN3*$^{-/-}$ mice exhibited altered intracellular Ca^{2+} handling with increased

SR Ca^{2+} leak and reuptake (Head et al. 2015). The reuptake of Ca^{2+} via the SERCA pump is an energy-consuming process that generates heat and hence, increased Ca^{2+} cycling may provide an advantage in cold environments (Head et al. 2015; de Meis 2001).

The aim of this study was to test whether humans with ACTN3 deficiency exhibit improved cold-tolerance and whether this is due to increased skeletal muscle Ca^{2+} cycling. 15 moderately active male subjects with the 577XX genotype (XX) and 27 control subjects with the normal genotype (577RR; RR) were included in the study. To test cold-tolerance, subjects were exposed to an acute cold-challenge consisting of intermittent whole-body immersion into cold (14°C) water. At baseline, there was no difference in rectal temperature (T_{re}), intramuscular temperature in the gastrocnemius muscle (T_{mu}), skin temperature (T_{sk}) and body composition between the two groups. During cold-water immersion T_{re} declined faster in RR than in XX subjects with a two times higher rate of temperature decline (Fig. 5A). The temperature decline in T_{mu} was also markedly faster in RR than XX subjects, whereas T_{sk} decline was similar in both groups. To assess whether improved cold-tolerance was caused by altered metabolic response to cold-water immersion, heart rate and respiratory rates were measured. During cold-water immersion, both, the rate of O_2 uptake and the rate of CO_2 production increased but the responses were similar in RR and XX subjects. The respiratory exchange rate decreased slightly during cold-water immersion and again, there was no difference in the response between the two groups. In both groups, the heart rate increased by ~40% and plasma levels of epinephrine and norepinephrine also increased similarly in both groups. In summary, our data suggests that humans lacking ACTN3 have a better cold-tolerance which is not caused by differences in body composition or by overall metabolic response to cold-water immersion.

During acute cold exposure, skeletal muscle generates heat by increased activation resulting in shivering. The extent of shivering was assessed from the EMG amplitude and frequency during cold-water immersion. There was no difference in EMG amplitude but the XX subjects had a lower average EMG frequency than the RR subjects. During prolonged cold exposure, shivering thermogenesis is replaced by non-shivering thermogenesis to prevent the muscle from fatigue and contractile dysfunction (Aydin et al. 2008). Our data suggests that shivering is not the cause of better cold-tolerance in the XX group and hence, non-shivering thermogenesis may be a contributing factor.

Increased Ca^{2+} cycling caused by SR Ca^{2+} leak and subsequent increased Ca^{2+} reuptake by SERCA provides a possible mechanism for heat generation (de Meis 2001). Dissociation of FKBP12 has previously been shown to result in leaky RyR1s and baseline muscle biopsies of both groups were therefore analysed for FKBP12 dissociation with RyR1 immunoprecipitation (Ahern et al. 1997). Both groups had similar FKBP12/RyR1 ratios suggesting that there was no FKBP12 dissociation and SR Ca^{2+} leak at rest in XX subjects at rest (Fig. 5B). FKBP12 dissociation and subsequent RyR1 leak are potentially induced by cold-exposure and analysis of biopsies taken during or immediately after cold-water immersion would be required to draw final conclusions.

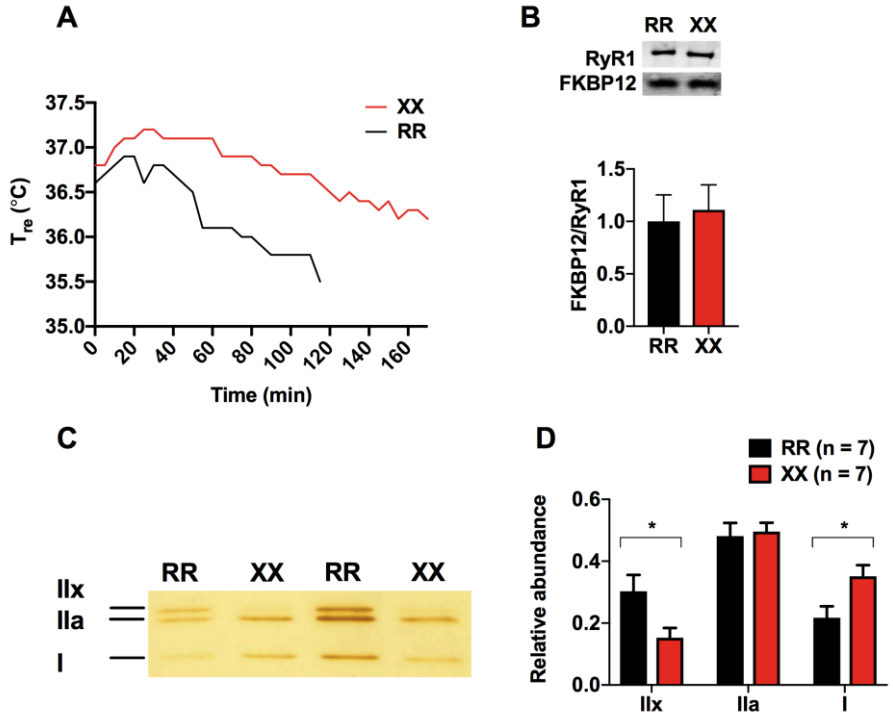

Figure 5. Increased cold-resilience in ACTN3 deficient humans is associated with altered fibre type composition. (A) Representative original records of rectal temperature (T_{re}) during cold-water immersion in an ACTN3 deficient (XX) and a control (RR) subject. **(B)** Representative blots (top) and summary data (bottom) of immunoprecipitated RyR1 in biopsies from RR and XX subjects **(C)** Representative image of silver-stained gels after electrophoresis for separation of myosin heavy chain isoforms in RR and XX subjects **(D)** Relative abundance of the different myosin heavy chain isoforms in RR and XX expressed relative to the total density of all bands in each subject. Bars represent mean ± SEM; *P < 0.05.

In the previously mentioned mouse study, *ACTN3*$^{-/-}$ mice had increased Ca^{2+} cycling, which was reflected in a higher abundance of SERCA1 and calsequestrin 1 (Head et al. 2015). Intriguingly, muscle biopsies of the XX subjects in our study had a decreased SERCA1 protein expression and an increased expression of calsequestrin 2 compared to the control group. SERCA1 and calsequestrin 1 are predominantly expressed in glycolytic type II muscle fibres whereas SERCA2a and calsequestrin 2 are mostly expressed in oxidative type I fibres and hence, the different expression of these proteins may simply reflect a difference in fibre type composition. Myosin heavy chain composition was analysed using electrophoresis and silver staining. The XX subjects had a significantly larger proportion of type I fibres and a reduced amount of IIx fibres compared to RR subjects whereas the proportion of type IIa fibres was similar in both groups (Fig. 5C-D). The silver staining method cannot provide information on the number of muscle fibres of each type and hence a decreased proportion of type IIx fibres may reflect a reduced number of type IIx fibres and/or a reduced cross-sectional area. Previous studies have reported a reduction in the cross-sectional area of type IIB and IIx fibres in mice and humans with ACTN3 deficiency, respectively (MacArthur et al. 2008; Broos et al. 2016).

Due to their increased oxidative metabolism and better fatigue-resistance, type I muscle fibres would be more effective for heat generation. This is also reflected in the EMG recordings of our study, which showed increased cold-tolerance despite lower frequency of thermoregulatory muscle tone during cold-exposure. In summary, our data suggests that humans with ACTN3 deficiency are better at maintaining their body temperature during acute cold-exposure which is likely explained by a larger proportion of type I muscle fibres.

4.4 EFFECT OF EXERCISE ON KYN METABOLISM (PAPER IV)

Exercise is generally recommended for patients with depressive disorder and a recent animal study has revealed a molecular pathway, which may mediate these beneficial effects via modulation of the KYN pathway. The degradation of KYN produces a number of neuroprotective and neurotoxic metabolites and imbalances in these metabolites have been associated with depression (Erhardt et al. 2017).

The recent discovery of enzymes of the KYN pathway (KATs) in skeletal muscle provided evidence for the cross-talk between muscle and brain. In mice, PGC-1α1, activated by endurance exercise, induced the expression of KATs in skeletal muscle, thereby shifting the peripheral KYN metabolism away from the neurotoxic branch towards the production of neuroprotective metabolites and hence protecting from depression (Agudelo et al. 2014). Some evidence suggests similar pathways in humans. A three-week endurance training program induced skeletal muscle KAT gene expression in untrained subjects, however, the effect on KAT protein expression in and on circulating KYN metabolites remain to be established (Agudelo et al. 2014).

The aims of this study were therefore to investigate the effect of exercise on peripheral KYN metabolism in humans. To test whether regular endurance exercise leads to adaptations of skeletal muscle KAT expression, muscle biopsies of endurance trained (END) and recreationally active but not specifically endurance trained (CTRL) subjects were analysed. Gene expression of all four KAT isoforms was significantly higher in the END group than in the CTRL group (Fig. 6C) and this corresponded to a higher protein expression of the four KAT isoforms (Fig. 6A). In addition, the mRNA expression of the upstream regulatory genes PGC-1α1 and PPARα were increased in the END group whereas gene expression of PPARδ was similar in the two groups. The END group also had increased expression of CPT1B, VEGFA and PDK4, all of which are downstream genes of the PGC-1α1 pathway. Consistent with the previously mentioned study, endurance training induced increased KAT gene expression (Agudelo et al. 2014), which was associated with an upregulation of the PGC-1α1 pathway.

To test the acute effect of different types of exercise on peripheral KYN metabolism, plasma concentrations of KYN metabolites were measured before and after endurance exercise (21 km run or 150 km cycling) and intense eccentric exercise consisting of 100 drop-jumps. 150 km of cycling caused a transient increase in plasma KYNA concentration 1 h after exercise that returned to baseline within 24 h (Fig. 6B). Plasma levels of QUIN also increased 1 h after exercise but to a lesser extent resulting in a decrease of the QUIN/KYNA

ratio. The same pattern was observed in the subjects who performed the 21 km run, where KYNA increased transiently by 125% (measured 30 min after the run) and returned to baseline levels 24 h later. In contrast, the 100 drop-jumps did not induce any changes in the circulating KYNA and QUIN levels, suggesting that changes in KYN metabolism are linked to energetically demanding exercise rather than high force eccentric contractions. Two possible explanations for this are *i*) that PGC-1α1 is induced by aerobic rather than resistance exercise (Lin et al. 2002; Ruas et al. 2012) and *ii*) that endurance exercise increases the availability of free tryptophan in the blood (Fernstrom and Fernstrom 2006). At rest, the majority of tryptophan is bound to albumin. Metabolically demanding exercise induces lipolysis and increases the concentration of free fatty acids in the blood, which bind competitively to albumin, thereby dissociating tryptophan and increasing the concentration of free tryptophan in the blood.

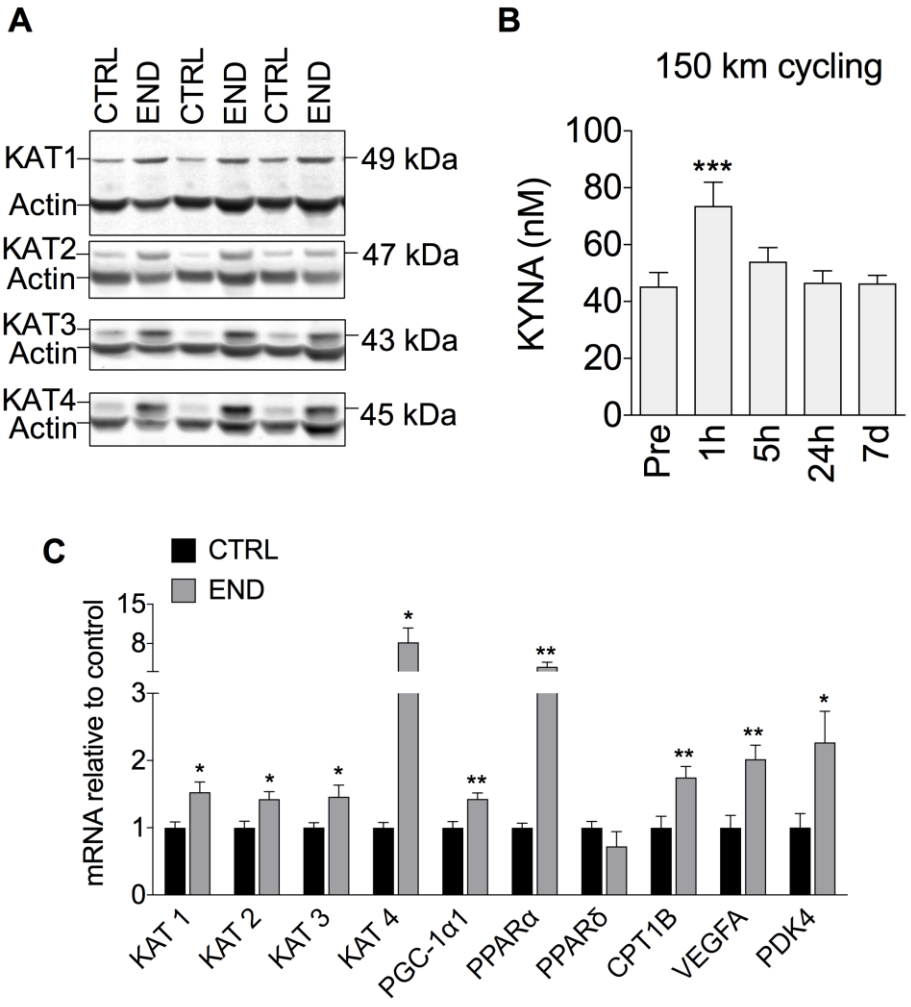

Figure 6. Effects of endurance training on skeletal muscle KAT gene and protein expression and on circulating KYNA concentration. (A) Western blots of different KAT isoforms from endurance trained (END) subjects and controls (CTRL). **(B)** Plasma KYNA concentration before and 1 h, 5 h, 24 h, and 7 days after participating in a 150-km road cycling time trial **(C)** Gene expression analysis from END and CTRL subjects. Bars represent mean ± SEM; *P < 0.05.

In summary, our study supports the mechanism of exercise-induced activation of PGC-1α1 and a subsequent increase in skeletal muscle KAT expression in humans. Prolonged aerobic exercise rather than exercise with high contractile force was required to induce changes in KYN metabolism and should therefore be recommended for patients with depression. Our study only included healthy subjects and follow-up studies including patients with depression are needed to complete the picture.

5 CONCLUDING REMARKS

The four papers presented in this thesis provide insights to several aspects of molecular adaptations of skeletal muscle in physiologic and pathologic conditions. In a contracting muscle fibre, Ca^{2+} is the main regulator of contractile force. In the linear part of the force-$[Ca^{2+}]_i$ relationship, small changes in $[Ca^{2+}]_i$ result in large changes of force. In paper I, we observed a force depression immediately and 1 h after SIT that was more pronounced at low than at high stimulation frequencies, suggesting reduced tetanic $[Ca^{2+}]_i$ and/or myofibrillar Ca^{2+} sensitivity as the underlying mechanism. Single muscle fibres stimulated with a SIT-mimicking protocol displayed SR Ca^{2+} leak and reduced tetanic $[Ca^{2+}]_i$ due to decreased availability of free Ca^{2+} in the SR (Place et al. 2015). Accordingly, we hypothesized that SIT would induce modifications of the RyR1 affecting SR Ca^{2+} release and tetanic $[Ca^{2+}]_i$ in untrained humans. We observed a SIT-induced reduction of the full-size RyR1 protein, however, this occurred 24 h after SIT when the contractile force was almost completely recovered. After a three-week training period, the RyR1 was partially protected from SIT-induced modifications while force depression was similar as in the untrained state. The RyR1 is a key player in muscle contraction and modifications, as seen 24 h after the first SIT session would be expected to impair EC coupling. However, our data does not confirm a causal relationship between modified RyR1 and contractile force. A possible explanation for the SIT-induced force depression is the accumulation of P_i, which inhibits the RyR1 and also reduces the availability of free Ca^{2+} in the SR due to Ca^{2+}-P_i precipitation (Allen et al. 2008b). The modification of the RyR1 on the other hand, may be caused by ROS, which accumulate in skeletal muscle during intense contractions (Powers and Jackson 2008). In line with this, SIT-induced fragmentation of the RyR1 was blocked by the application of antioxidants in previous experiments on isolated mouse toe muscles (Place et al. 2015). The RyR1 is susceptible to ROS-modifications and previous studies have shown ROS-induced FKBP12 dissociation and subsequent RyR1 leak (Andersson et al. 2011). Training increases the antioxidant capacity in muscles and that could explain why the RyR1 was partially protected from SIT-induced modifications in the trained state (Criswell et al. 1993; Powers et al. 1994). However, it remains unclear how EC coupling can be intact despite biochemical changes of the RyR1. Only every other RyR1 is coupled with a DHPR and the activation of the uncoupled RyR1s is incompletely understood. Potentially, SIT only affects the uncoupled RyR1s thereby preserving EC coupling in the remaining channels. Due to limited biopsy material, we could not perform additional experiments on our samples but further studies investigating FKBP12 dissociation, Ca^{2+} handling proteins as well as antioxidant enzymes after SIT may improve our understanding of the observed RyR1 modifications.

In the second paper, we investigated adaptations of skeletal muscle to neurodegenerative disease leading to atrophy and muscle weakness in a mouse model of ALS. Surviving muscle fibres of ALS mice exhibited intact intracellular Ca^{2+} handling and preserved force generation and we can therefore confirm that muscle weakness is primarily caused by denervation and subsequent atrophy and not by muscle fibre intrinsic defects. Other studies have reported impaired contractile function in the same mouse model and it has

been hypothesized that these defects are caused by cellular toxicity due to accumulation of mutated SOD1 protein (Dobrowolny et al. 2008; Wong and Martin 2010). During single fibre dissection, we electrically stimulate the muscle to ensure that we perform the experiment on a contracting fibre. It is possible, that we introduce some bias during this step, by excluding fibres that contract less and hence would have displayed impaired contractility. We have not measured SOD1 aggregates and further studies may shed light on the existence of these protein accumulations and their impact on contractile function. The surviving muscle fibres in our study showed endurance training-like adaptations with increased fatigue-resistance and signs of mitochondrial biogenesis. Most likely, this is a compensatory adaptation, as the surviving fibres have to take over the tasks of the fibres that are no longer innervated. Our data shows that even in the late stage of the disease, innervated muscle fibres maintain their ability to improve their fatigue-resistance, which might be of use for training interventions or medical treatment of ALS. It should be noted, that the mouse model we used only represents a minor proportion of all ALS patients and future studies using different mouse models as well as clinical studies on ALS patients would add valuable knowledge.

In paper III, we demonstrate how altered expression of a structural protein in muscle fibres can affect whole body physiology and thermoregulation in humans. Subjects lacking ACTN3 had improved cold-resilience compared to control subjects when exposed to an acute cold-water challenge. Skeletal muscle of mice lacking ACTN3 display increased intracellular Ca^{2+} cycling, which requires increased hydrolysis of ATP to drive the SERCA pump (Head et al. 2015). This could be a potential mechanism for increased heat-generation and better cold-tolerance in ACTN3 deficiency. We tested muscle biopsies of our subjects for dissociation of FKBP12 from the RyR1, which results in leaky RyR1 (Bellinger et al. 2008). A chronic Ca^{2+} leak from the SR would cause pathologic elevations in resting $[Ca^{2+}]_i$ and hence, we would expect increased SERCA pump activity to counteract the rise in resting $[Ca^{2+}]_i$. However, there was no difference in the FKBP12/RyR1 association in resting biopsies from the two groups, suggesting that there is no RyR1 leak at rest, which is also in agreement with the baseline body temperature that was not different between the two groups. It has been stated that ACTN3 deficiency has undergone positive selection in recent evolution when areas with cold temperature and scarce food sources were inhabited and that ACTN3 deficiency would provide a survival advantage in these areas (MacArthur et al. 2007). From that perspective, chronically increased Ca^{2+} cycling would generate heat but would also require more energy when food is a limiting factor. It remains to be established whether increased Ca^{2+} cycling and heat-generation need cold exposure and this would require the analysis of biopsies collected during or directly after cold-water immersion. We have found an increased proportion of type I and a decreased proportion of type IIx muscle fibres in the ACTN3 deficient subjects. More oxidative fibres would be more energy-efficient, which would fit to the hypothesis of better survival in areas with scarce food sources. In addition, a larger proportion of type I fibres would also provide advantage for endurance exercise, which is in line with the observation that the 577XX genotype is overrepresented in elite endurance athletes (Yang et al. 2003). Our data suggest that type I fibres are more efficient at generating heat during acute cold-exposure, which might be related to the fact that motor units driving

type I fibres are more prone to increased muscle tone rather than shivering and this may be a more effective way of protecting body temperature (Lomo et al. 2019). Biochemical analyses performed on single muscle fibres instead of muscle homogenates would provide valuable insights to specific adaptations in the individual fibre types. ACTN3 is mainly expressed in fast, glycolytic fibres and it is possible that the lack of ACTN3 causes some dysfunction that is compensated by an increase in type I muscle fibres and this in turn provides survival advantage in cold areas and improved endurance performance.

In paper IV, we show that endurance exercise induces skeletal muscle adaptations, which may have implications for peripheral KYN metabolism and the cross-talk between muscle and brain. Increased skeletal muscle KAT expression has been associated with a shift in the peripheral KYN metabolism and has been shown to protect from stress-induced depression in mice (Agudelo et al. 2014). Our data confirms that regular endurance exercise induces the PGC-1α1 pathway and skeletal muscle KAT expression also in humans and that acute endurance exercise increases KYNA levels in the blood. It should be noted that the subjects that performed the 150 km cycling and 21 km running exercise in this study had prepared for these events and were therefore endurance trained. Hence, it remains to be established whether the transient increase in KYNA is related to acute endurance exercise or to the increased skeletal muscle KAT expression in endurance trained subjects. Either way, endurance exercise activates peripheral KYN metabolism, which leads to chronic adaptations in skeletal muscle that seem beneficial for depressive disorder. High-intensity eccentric exercise, on the other hand, did not increase KYNA levels in the blood and would therefore unlikely induce chronic adaptations. This is in agreement with the fact that PGC-1α1 is specifically induced by endurance exercise, whereas resistance exercise induces PGC-1α2, 3 and 4 (Lin et al. 2002; Ruas et al. 2012). Our study included only healthy subjects and future studies should aim at investigating skeletal muscle KAT expression as well as the effect of endurance exercise on circulating KYNA in patients with depression who display chronic imbalances in the KYN metabolites. Depression is a multifaceted disease. The link between endurance exercise and peripheral KYN metabolism provides a promising alternative to directly target depression with exercise interventions. Moreover, this type of therapy is free from side effects and has other beneficial effects on general health.

Skeletal muscle is a multifaceted tissue that adapts to changing environments and altered external stimuli with molecular changes that can have both, local and systemic effects. The four studies presented in this thesis underline the adaptability of skeletal muscle to several types of exercise, to chronic disuse during disease and to altered genetic predisposition.

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